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Environmental Signals Controlling Sexual Development of the Corn Smut Fungus *Ustilago maydis* through the Transcriptional Regulator Prf1

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Environmental signals induce and coordinate discrete morphological transitions during sexual development of *Ustilago maydis.* In this fungus, mating of two compatible haploid sporidia is a prerequisite for plant infection. Cell fusion is governed by the action of pheromones and receptors, whereas the subsequent pathogenicity program is controlled by the combinatorial interaction of homeodomain proteins. The *U. maydis* pheromone response factor (Prf1) is a central regulator of both processes. We have analyzed the regulation of the *prf1* gene and demonstrate that pheromone and cAMP signaling regulate *prf1* post-transcriptionally. Transcriptional activation of *prf1* was observed in the presence of carbon sources, such as glucose and fructose, allowing us to define the *cis*-acting element in the *prf1* promoter that mediates these effects. The same element provides for negative control of *prf1* gene transcription at high cAMP levels. A protein that specifically binds to this element was purified and analyzed for its role in *prf1* gene regulation. On the basis of these results, we present a model in which *prf1* integrates different environmental signals to control development in *U. maydis*.

INTRODUCTION

Plant pathogenic fungi must recognize susceptible hosts, overcome physical and chemical barriers, and colonize plant organs to exploit available nutrients. To survive, most fungi have evolved specialized cell types, such as appressoria, infection hyphae, or haustoria. The formation of these structures appears to be tightly controlled and depends on the perception of signals from the environment. Elucidating the nature of these signals and how they are transduced remains an important challenge in phytopathology.

In the corn smut fungus *Ustilago maydis*, sexual development is controlled genetically by two unlinked mating-type loci, termed *a* and *b* (Rowell, 1955; Holliday, 1961; Banuett, 1995). The *a* locus, which exists in the two alleles *a1* and *a2*, controls fusion of haploid cells. Each allele contains genes coding for a pheromone precursor (*mfa*) and a pheromone receptor (*pra*), recognizing pheromones of the opposite mating type (Bölker et al., 1992). After cell fusion, a filamentous dikaryon, which is able to infect the plant, is formed. This differentiation requires the activity of the *b* locus, which is multiallelic and encodes a pair of homeodomain proteins, termed bW and bE (Gillissen et al., 1992). In nonallelic combinations, these proteins can form heterodimers and initiate the pathogenicity program (Kämper et al., 1995).

The expression of the mating-type genes, for example, the pheromone and receptor genes as well as the *bE* and *bW* genes, is regulated by various signals. In haploid sporidia, these genes are expressed at a low level, depending on the medium used for growth (Spellig et al., 1994). During mating, these genes are upregulated through pheromone stimulation. After cell fusion, the bE/bW heterodimer appears to repress transcription of the pheromone and receptor genes only (Urban et al., 1996). The high mobility group domain protein Prf1 (for pheromone response factor) directly controls expression of the mating-type genes by binding to a short DNA sequence (pheromone response element [PRE]), present in the *a* and *b* mating-type loci (Hartmann et al., 1996; Urban et al., 1996). In *prf1* mutant strains, these genes are poorly transcribed, even under conditions of pheromone stimulation. Consequently, *prf1* mutants are sterile and nonpathogenic. Pathogenicity can be restored, however, by overexpression of an active bE/bW heterodimer, indicating that activation of *b* gene transcription is the key function of *prf1* during pathogenesis (Hartmann et al., 1996).

Cell fusion and pathogenicity also are influenced by a variety of environmental signals, which hitherto have not been defined clearly. Cell fusion, for example, which normally may occur in the leaf whorl, can be monitored on plates but requires conditions of starvation or the presence of activated

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charcoal (Day and Anagnostakis, 1971; Snetselaar, 1993; Banuett and Herskowitz, 1994). In addition, prolonged growth and mitotic cell division of the dikaryon can be observed only after penetration of the host tissue, indicating the need for some type of signal(s) provided by the plant.

Recent work has indicated that one of these signals could act via the cAMP pathway (Gold et al., 1997; Krüger et al., 1998). Components of this signal transduction pathway originally were identified in *U. maydis* due to their influence on morphology. The *uac1* gene, encoding adenylate cyclase, was isolated via a mutation that confers a constitutive filamentous growth habit on haploid cells (Gold et al., 1994). The *ubc1* gene, encoding a regulatory subunit of a cAMP-dependent protein kinase (PKA; Taylor et al., 1990), was identified as a suppressor of the phenotype of the *uac1* mutant.

Recently, Gold et al. (1997) investigated the influence of a *ubc1* mutation on pathogenicity of *U. maydis.* They reported that coinoculation of maize seedlings with compatible *ubc1* mutant strains resulted in initial pathogenicity symptoms, such as anthocyanin synthesis and chlorosis. However, the mutants were unable to induce plant tumors, indicating the need for cAMP signaling during pathogenesis. Interestingly, the cAMP pathway also is involved in the regulation of pheromone gene expression. It was shown that the G protein α subunit Gpa3 acts by stimulating adenylate cyclase and that this pathway is essential for mating and pheromone gene induction in *U. maydis* (Regenfelder et al., 1997; Krüger et al., 1998).

Considering the central position of Prf1 in the regulation of the *a* and *b* mating-type genes, it is conceivable that Prf1 receives signals from the environment. To test this hypothesis, we have analyzed the regulation of *prf1* with respect to different extracellular and intracellular signals. In this study, we show that the *prf1* gene is regulated in a complex manner, both transcriptionally and post-transcriptionally. Transcriptional control occurs through a *cis*-regulatory DNA element that mediates two functions—it confers gene induction at high glucose concentrations, and it provides for gene repression at high internal cAMP levels. In addition, we report the isolation of a novel DNA binding protein that participates in the transcriptional control of *prf1*.

RESULTS

prf1 and *mfa1* Are Coordinately Regulated and Respond to the Pheromone as Well as the cAMP Pathway

Because PREs are present in the promoter regions of *prf1* and *mfa1*, it was assumed that both genes are regulated by Prf1 (Hartmann et al., 1996). To test this assumption, we conducted RNA gel blot analyses with these two genes from different strains.

In a haploid wild-type strain, FB1, both genes showed a low basal level of expression (Figure 1, lane 1). To investigate the effect of an activated pheromone-signaling cascade, we used strain FB1pra2, which carries the receptor of the opposite mating type and is capable of autostimulation. Compared with FB1, this strain showed an induced level of transcription of both genes (Figure 1, lane 4). Pheromone induction also was observed at an early time point in the mating reaction (Figure 1, lane 5), whereas at a later time point, when most of the cells had fused, the bE/bW heterodimer inhibited expression of both genes (Figure 1, lane 6; Urban et al., 1996). When comparing lanes 4 and 5 (Figure 1), one has to take into account that during the mating reaction (Figure 1, lane 5), only 50% of the cells express the *mfa1* pheromone gene, whereas the remaining 50% express the *mfa2* gene. In lane 4 (Figure 1), no mating partner is present. With respect to *prf1*, the signal in lane 5 (Figure 1) resulted from both mating partners.

When the regulatory subunit of PKA, *ubc1*, was deleted, the resulting constitutive activation of the pathway induced transcription of both *mfa1* and *prf1* (Figure 1, lane 3). Conversely, a deletion of the adenylate cyclase gene *uac1* that interrupts the pathway reduces the level of transcription of both genes (Figure 1, lane 2). These results demonstrate that transcription of *prf1* and *mfa1* is coordinated under all conditions tested and is influenced by pheromone as well as by cAMP signaling and the b heterodimer. Because *prf1* and *mfa1* contain PREs in their respective promoter regions, and because the *mfa1* gene promoter has been shown to bind

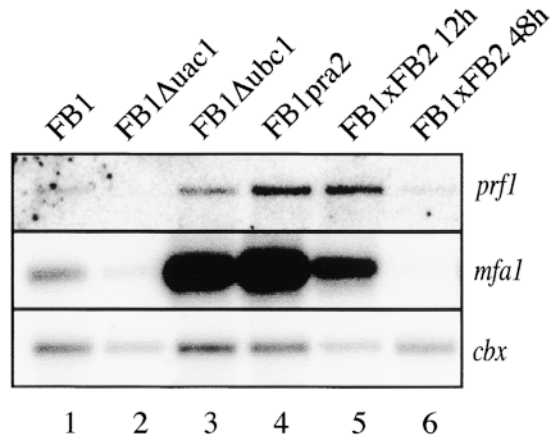

Figure 1. Regulation of *prf1* and *mfa1* Gene Transcription.

RNA was prepared from the strains indicated at top. Strains were grown for 48 hr (48h) on complete medium charcoal plates, except where indicated otherwise. Fifteen micrograms of total RNA was loaded per lane. The filter was hybridized in succession with the gene probes for the *prf1* gene, the *mfa1* gene, and the constitutive succinate dehydrogenase control (*cbx*).

Prf1, the observed coordinate regulation is likely to be exerted by Prf1 through autoregulation (Hartmann et al., 1996).

Identification of a *cis*-Regulatory Element in the *prf1* Promoter

The *prf1* gene promoter contains two PREs located between positions −729 and −706. They are most likely the elements for autoregulation of *prf1* gene transcription (Hartmann et al., 1996). To define functional *cis*-regulatory elements in the *prf1* promoter region, we cloned the *prf1* gene, including different parts of the 5′ region, into a free-replicating plasmid and transformed it into the *prf1* mutant strain HA99 (*a1 b1 Δprf1-1*). Plasmids expressing *prf1* should be able to complement the mating and pheromone production defects of the *prf1* mutant strain. This possibility was tested by cospotting the transformants with appropriate tester strains on charcoal-containing plates, as shown in Figure 2A. Surprisingly, a plasmid containing 1.4 kb of upstream sequence (pRFΔ$P_{1.4}$; Figure 2A) was not able to complement, whereas mating and pheromone production were restored when the 5′ region was extended to 1.6 kb (plasmid pRFΔ$P_{1.6}$; Figure 2A). This indicates the existence of a *cis*-regulatory element in this region, which does not coincide with the PREs.

This region was delineated further by creating a series of nested deletions, resulting in an 85-bp fragment in the *prf1* gene promoter extending from −1594 to −1509, termed the upstream activating sequence (UAS), which was necessary for *prf1* expression (see Methods). To further analyze the function of the UAS, we generated reporter gene fusions. These fusions contained the green fluorescent protein (GFP)–encoding gene behind a minimal promoter derived from the *mfa1* gene. This promoter did not allow expression of GFP on its own (plasmid pmfa–sgfpE; Figure 2B). The UAS was inserted into the reporter gene construct at two different locations, in both orientations, and at different copy number, as shown in Figure 2B.

The various plasmids were linearized and integrated ectopically into the *U. maydis* genome. For each construct, six independent transformants were analyzed microscopically for green fluorescence. We observed that ∼40% of the transformants showed green fluorescence when grown on PD plates (see Methods), irrespective of the orientation or the site at which the UAS had been inserted. Transformants generated with plasmids pE–UAS, pE–UASi, and pB–UAS showed less fluorescence than did transformants harboring pB–UAS2i and pB–UAS3, which contain multiple copies of the UAS (Figure 2B). Interestingly, all transformants that showed fluorescence when grown in PD medium (see Methods) expressed significantly less GFP when propagated in YEPS medium (see Methods; Figure 2B). Using synthetic oligonucleotides, we further delineated the UAS to a 40-bp fragment, which was active in promoting GFP expression (plasmid pB–UAS40; Figures 2B and 2C). Taken together, these results show that the UAS identified in the *prf1* gene promoter is crucial for promoter activity and confers medium-dependent expression to a reporter gene.

Nutrients and cAMP Control *prf1* Expression via the UAS

The reporter gene constructs allowed a detailed analysis of the medium compounds regulating activity of the UAS. Strain HA232, carrying pB–UAS3 stably integrated into its genome, was precultured in YEPS medium, washed with water, and diluted 500-fold into various culture media (Table 1). Green fluorescence was quantified after the cultures had reached an $OD_{600\ nm}$ of 1.0. Fluorescence was detected after growth in PD broth as well as in minimal medium, with glucose as the sole carbon source. In YEPS medium, green fluorescence was approximately fivefold lower than in minimal glucose medium (Table 1). Next, we investigated the influence of different carbon sources on expression of the reporter gene in minimal medium. Fluorescence measurements revealed that glucose, fructose, sucrose, and arabinose were equally effective in inducing expression. Xylose caused a small but significant induction, whereas maltose, ethanol, or glycerol had no effect (Table 1). On minimal glucose medium and minimal maltose medium, growth rates of the strains were comparable (data not shown). Induction could be observed within 2 hr after transfer from YEPS to minimal glucose medium, indicating that induction was caused by the sensing of carbon source. In minimal glucose medium, the addition of cAMP led to a decrease in reporter gene activity, which correlated with the amount of cAMP added (Table 1). Similar results were obtained with strain HA185 (*a2 b2* B-UAS3), which carries the same reporter gene construct integrated at a different genomic location (data not shown).

To confirm the results obtained with the reporter gene fusion, we conducted RNA gel blot analysis with strain FB1 to investigate directly the influence of maltose and glucose on the expression of the *mfa1* gene (Figure 3). The *mfa1* gene was chosen for these experiments because the transcript is more abundant than the *prf1* gene transcript. The results were fully consistent with the findings obtained using the UAS–reporter gene constructs. In minimal glucose medium, transcription of *mfa1* was induced, whereas in minimal maltose medium, *mfa1* was not transcribed. These results illustrate that the UAS has a dual role and provides for glucose induction as well as for cAMP repression of *mfa1* via Prf1.

Isolation of a UAS Binding Protein

To identify potential transcription factors binding to the *cis*-regulatory element, we conducted gel retardation experiments by using crude protein extracts from strain FB1 (*a1 b1*) grown under inducing (minimal glucose medium) and

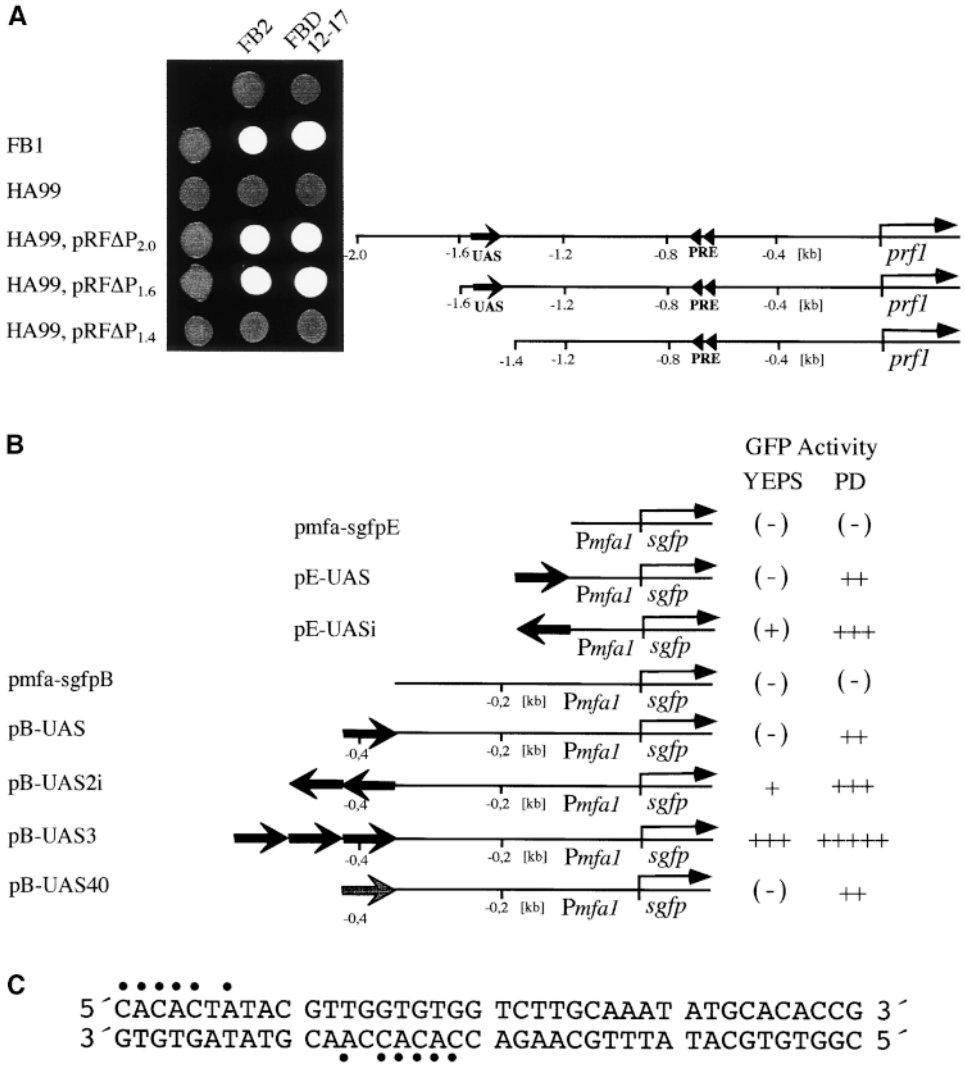

Figure 2. Identification of a UAS.

(A) Complementation of a *prf1* mutant strain. The *prf1* mutant strain HA99 (*a1 b1 Δprf1-1*) was transformed with plasmids containing different parts of the *prf1* promoter, as indicated at right. Mating and pheromone production were assayed by co-spotting the transformants with strains FB2 (*a2 b2*) and FBD12-17 (*a2 a2 b1 b2*) on PD charcoal plates. The appearance of a white filament indicates complementation.

(B) The UAS confers medium-dependent expression to a reporter gene. The reporter gene constructs indicated at left, consisting of a minimal promoter (P*mfa1*) fused to the green fluorescent protein (*sgfp*) gene and the indicated UAS fragments, were transformed into *U. maydis* strain FB2, and green fluorescence was assayed microscopically after overnight growth in YEPS and PD medium, respectively. (−) indicates that no fluorescence could be detected; (+) to +++++, increasing levels of fluorescence. The filled arrows represent the 85-bp fragment containing the UAS, except in pB-UAS40, where the arrow indicates the 40-bp UAS fragment, whose sequence is depicted in **(C)**. The bent arrows represent the transcription initiation points of the reporter gene.

(C) DNA sequence of the UAS. Minimal sequence of the UAS that was active promoting reporter gene expression in *U. maydis.* Dots indicate a palindromic sequence in the element.

noninducing (minimal maltose medium) conditions. In both extracts, a protein (or protein complex) could be identified that showed specific binding to the 85-bp UAS-containing DNA fragment, as shown in Figure 4A. The band shift provided the assay to follow UAS binding activity during subsequent purification. Crude extract was first loaded onto a heparin–Sepharose column and eluted using a continuous salt gradient. Activity eluted at a concentration between 0.6 and 0.8 M NaCl, which is indicative of strong binding involving polar interactions. The active protein fraction was puri-

fied further on a Mono Q anion exchange column, again using a continuous salt gradient for elution. Comparison of the protein profiles in fractions with high and low UAS binding activity allowed us to identify two candidate proteins with a molecular mass of ~60 kD (Figure 4B).

Because the genuine N termini of both proteins were blocked, both proteins were digested with the endoproteinase Lys-C, which resulted in an almost identical peptide pattern (data not shown). Therefore, we assumed that these two proteins were likely to be products of the same gene and that their different migration in SDS gels reflected either dissimilar post-translational modification or degradation. From three of the peptides derived from the smaller protein, partial amino acid sequences were determined by Edman degradation (KT/YAADLAK, AVEDLEIK, and A/QVNDQARSR-QAAELHSARR). On the basis of these peptide sequences, degenerate oligonucleotide primers were synthesized and used in combination with a vector primer in a polymerase chain reaction with a cDNA library as a template. One amplified fragment had the potential to encode all three peptide sequences, and this fragment was used to isolate genomic and cDNA clones. Sequencing revealed that the complete 400–amino acid protein, with a calculated molecular mass of 40 kD, was encoded on a single exon (Figure 5A). We consider the difference between the calculated molecular mass and the observed migration in SDS-containing polyacrylamide gels (60 kD; Figure 4B) likely to result from post-translational modification. The predicted amino acid sequence of the protein contains three potential N-glycosylation sites at amino acid positions 7, 21, and 39, respectively.

Table 1. Influence of Growth Media on UAS Activity in Strain HA232

Growth Medium[a]	Relative Fluorescence[b]
YEPS	17 (±4)
PD	162 (±34)
1% D-(+)-Glucose (MM)	100
1% D-(−)-Fructose (MM)	103 (±12)
1% L-(+)-Arabinose (MM)	98 (±12)
1% Sucrose (MM)	91 (±12)
1% D-(+)-Xylose (MM)	32 (±6)
1% Maltose (MM)	18 (±4)
1% Ethanol[c] (MM)	18 (±4)
1% Glycerol[c] (MM)	12 (±4)
Glucose (MM), 3 mM cAMP	63 (±5)
Glucose (MM), 6 mM cAMP	47 (±4)
Glucose (MM), 15 mM cAMP	13 (±3)

[a] MM, minimal medium.

[b] Strains were grown to an $OD_{600\ nm}$ of 1.0, and relative fluorescence was determined. Fluorescence observed when grown on minimal glucose medium was set to 100. Given are the average values of three independent experiments. Numbers within parentheses denote the standard deviation.

[c] These carbon sources led to significantly slower growth.

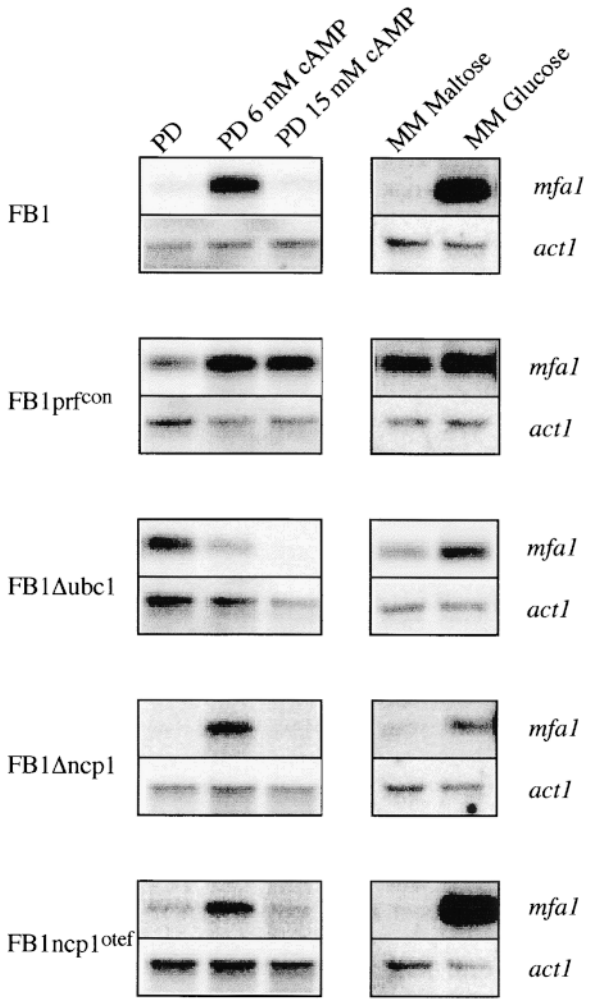

Figure 3. Control of Pheromone Gene Expression by cAMP and Carbon Source.

Expression of the pheromone gene *mfa1* was determined by RNA gel blot analysis. Strains were grown in PD broth with the indicated level of cAMP (left) and in minimal medium (MM) with glucose or maltose as the sole carbon source (right). Relevant strain genotypes are indicated at left. Ten micrograms of total RNA was loaded per lane. The filters were hybridized in succession with the gene probes for the *mfa1* gene and the constitutive actin control (*act1*).

However, we cannot exclude anomalous migration behavior due to special structural features.

The region comprising the presumed translational start site (TCACCATGAC; underlined) conforms to the fungal consensus sequence for translation initiation (Ballance, 1990). Unexpectedly, we could not detect any significant similarity to known proteins or protein domains by searching the GenBank and EMBL databases, except for a monopartite nuclear localization sequence (KKRH) at position 355.

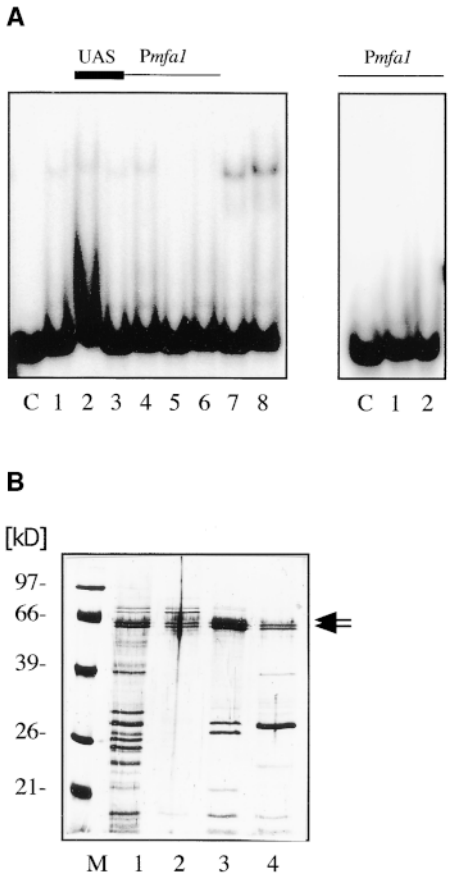

Figure 4. Isolation of a UAS Binding Protein.

(A) Gel retardation assay. Protein extracts were prepared from strains FB1 (*a1 b1*; lanes 1 to 4), HA282 (*a1 b1 Δncp1*; lanes 5 and 6), and HA296 (*a1 b1 ncp1*^{otef}; lanes 7 and 8). Strains were grown in glucose minimal medium (lanes 1, 2, and 4 to 8) and maltose minimal medium (lanes 3 and 4) to an $OD_{600\ nm}$ of 0.8. Ten micrograms (lanes 1, 3, 5, and 7) and 20 μg (lanes 2, 4, 6, and 8) of crude protein extracts were used in the binding reactions. In lane C, no protein was added. The DNA fragments used are indicated at top.
(B) Partial purification of the Ncp1 protein. Protein extracts after heparin–Sepharose (lane 1) and anion exchange chromatography (lanes 2 to 4) were separated on a 12% SDS–polyacrylamide gel and silver stained. Lane 3 contains the fraction with the highest specific UAS binding activity, whereas lanes 2 and 4 are the border fractions. Lane M contains molecular mass markers, as given at left in kilodaltons. The arrows indicate candidate proteins whose presence correlates with band shift activity.

Figure 5B shows that the protein can be divided into a mostly uncharged N-terminal part and a highly charged C-terminal domain. According to its proposed function, the gene was named *ncp1* (for nutritional control of *prf1*; GenBank accession number AF082296).

Ncp1 Participates in the Induction of *prf1*

To elucidate the function of the *ncp1* gene, we generated null mutants in strains HA232 (*a2 b2 B-UAS3*) and CL13 (*a1 bW2 bE1*) by replacing the complete open reading frame with a hygromycin resistance cassette. DNA gel blot analysis was used to verify that gene replacement had occurred, and this provided no hints to the existence of a second gene related in sequence to *ncp1* (data not shown). Surprisingly, deletion of the *ncp1* gene in CL13 did not lead to any defect with respect to growth, morphology, or pathogenicity (data not shown). Strain HA263 (HA232*Δncp1*) still was able to fuse and form tumors in combination with a compatible wild-type strain (FB1). From the progeny of this infection, strain HA282 (*a1 b1 Δncp1*) was selected, carrying the mutant allele in an *a1 b1* background. In combination, strains HA263 and HA282 were able to mate and initiate plant tumor development at an efficiency not significantly different from that of the wild type (data not shown). Furthermore, induction of the reporter gene construct by glucose in strain HA263 was not altered compared with HA232 (data not shown). Nevertheless, in a band shift assay using protein extracts from strain HA282, no UAS binding activity could be detected (Figure 4A, lanes 5 and 6), confirming that the

A

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MTDLARNGSI LVAEIKAIHP ANGSDATNGA VPHANGDANG  40
SAILSKDAPT LPATAGAGAA APIESSITDP ATQPHLGNGH  80
AHQATDLSIG AGAVAVPHQA DTSVASGIAD PVAQSPAPTA 120
TDAAAPGLVQ PQTTAIQPVQ PNDHAREVSH INTAVPAAGV 160
GAAAATAAPG SAVEPTSAVS AVAPSTAPGS AISAQQPQQL 200
NAVASSPTAA GAPSFPPGTI VPSSVTGAGA GAGAGAGAGA 240
GAATVAPIVA HPPPTTLAAT EAPKSALSPK EAKKMDKLMK 280
KEAKNEHKSL KRAIKDAAAS EKLFRKSRDA ETQALKRHQK 320
AQTKEHKAAK ALSKVKAEYE KYAADLAKAV EDLEIKKRHT 360
ESTREGYETS KKHIDELRNQ KTVNDQARSR QAAELHSARR 400
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B

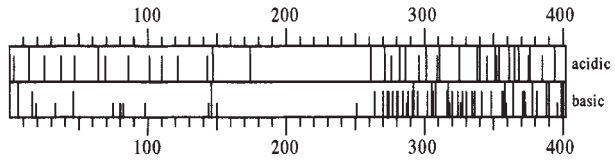

Figure 5. Amino Acid Sequence of the Ncp1 Protein.

(A) The deduced amino acid sequence of the Ncp1 protein.
(B) Distribution of charged amino acid residues in Ncp1.

Ncp1 protein is responsible for the band shift observed in wild-type strains.

Next, we investigated whether the deletion or the overexpression of *ncp1* had any influence on the pattern of pheromone gene regulation. Overexpression of *ncp1* was achieved by fusing the gene to the strong *otef* promoter (Spellig et al., 1996) and targeting the construct (plasmid pncp1^{otef}) to the genomic *cbx* locus, which encodes a subunit of succinate dehydrogenase (Keon et al., 1991). In a protein extract isolated from strain HA296 (*a1 b1 ncp1*^{otef}), approximately threefold more band shifting activity was detected compared with extract from wild-type cells (Figure 4A, lanes 7 and 8), indicating that Ncp1 is indeed overproduced. Next, RNA was isolated from strains HA282 (*Δncp1*) and HA296 (*a1 b1 ncp1*^{otef}), grown in minimal glucose or minimal maltose medium and in PD medium supplemented with cAMP (Figure 3). Both strains showed normal regulation (see below) of the *mfa1* gene with respect to carbon source and cAMP, although expression levels in minimal glucose medium were reduced approximately sevenfold in the *ncp1* mutant strain and increased approximately twofold in the overexpressing strain compared with the wild type. This indicated that *ncp1* plays a positive but modest role in *prf1* gene regulation.

prf1 Is Subject to Both Transcriptional and Post-Transcriptional Regulation

Studies with the UAS had indicated that media as well as cAMP affect *prf1* gene expression. We next decided to establish a system whereby transcriptional and post-transcriptional effects could be distinguished. However, due to the autoregulatory control mechanism, it was not possible to assess whether a signal directly affects transcription of the *prf1* gene or modulates Prf1 protein activity. To resolve this problem, we constructed a new *prf1* allele (*prf1*^{con}), where the *prf1* gene is transcribed from the constitutive *tef1* promoter, as shown in Figure 6A. This allele was introduced into haploid *U. maydis* strains in such a way that it replaced the resident *prf1* allele. The resulting strains HA271 (*a1 b1 prf1*^{con}) and HA266 (*a2 b2 prf1*^{con}) showed no obvious phenotype regarding morphology, mating, and pathogenicity, indicating that the *prf1*^{con} allele was functional and that constitutive expression of *prf1* did not interfere with normal development of *U. maydis* (data not shown). We noticed, however, that HA271 and HA266 could fuse and form dikaryotic filaments on media like YEPS, for which respective wild-type strains were unable to initiate this developmental program (data not shown).

Strains FB1 (*a1 b1*) and HA271 (*a1 b1 prf1*^{con}) subsequently were used to monitor *mfa1* gene expression after exposure to different stimuli. The rationale for these experiments was as follows. Conditions that lead to an alteration in pheromone gene expression in HA271 were considered to act at a post-transcriptional level on Prf1. In contrast, conditions that have an effect on pheromone gene transcription in the wild-type strain FB1 but that do not show this effect in HA271 must act on the transcriptional control of *prf1*.

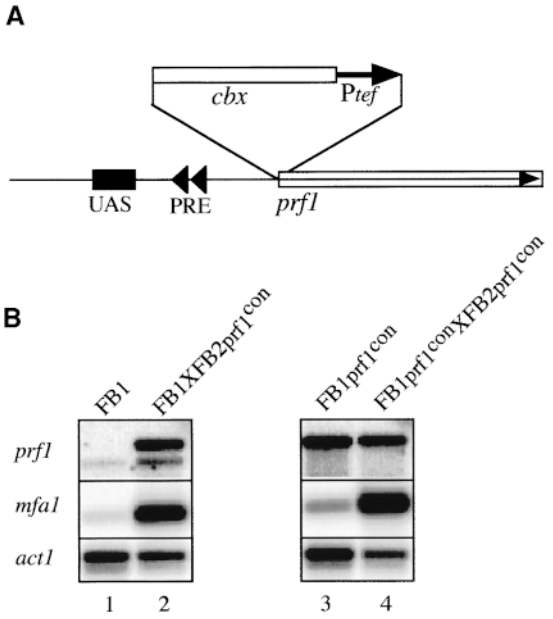

Figure 6. Constitutive Expression of *prf1*.

(A) The *prf1*^{con} allele is shown schematically. The *cbx* resistance gene, flanked by the constitutive *tef1* promoter (P_{tef}), was inserted in front of the *prf1* gene. The locations of the PRE and a UAS in the *prf1* promoter are indicated.

(B) Effects of the *prf1*^{con} allele on the expression of the pheromone gene *mfa1* are analyzed by RNA gel blot analysis. Strains FB1 (*a1 b1*) and FB1prf1^{con} (*a1 b1 prf1*^{con}) were grown on complete medium charcoal plates alone (lanes 1 and 3) or cocultivated with strain FB1prf2^{con} (*a2 b1 prf1*^{con}) to achieve pheromone stimulation (lanes 2 and 4). The filter was hybridized in succession with probes for *prf1*, *mfa1*, and *act1*, as indicated at left. The larger signal detected with the *prf1* probe represents transcripts from the *prf1*^{con} allele.

The expression of the *prf1*^{con} allele led to a transcript with a lower electrophoretic mobility when compared with the wild-type transcript, probably because a different transcription initiation site is used in the *tef1* promoter construct (Figure 6B). As expected, the amount of *prf1*^{con} transcript in strain HA271 was comparable under the conditions tested, showing that the activity of the *tef1* promoter was indeed constitutive and not influenced by the factors investigated. The basal expression of the *mfa1* gene was approximately twofold higher in strain HA271 than in the wild-type strain, which reflects differences in promoter activity (Figure 6B, lanes 1 and 3). Interestingly, *mfa1* expression in strain HA271 still could be induced by pheromone stimulation, which shows that the pheromone signal activates Prf1 at a post-transcriptional level. In contrast, regulation by carbon source

no longer could be observed, confirming that this signal acts on *prf1* transcription (see Figure 3).

prf1 Is Controlled by Divergent cAMP Pathways

To investigate the effects of cAMP on *prf1* activity, we cultivated strains FB1 and HA271 in liquid medium with different levels of cAMP (Figure 3). As observed previously (Krüger et al., 1998), the wild-type strain FB1 showed an induction of pheromone gene expression in the presence of 6 mM external cAMP, whereas transcription dropped when higher amounts of cAMP (15 mM) were added. In strain HA271, similar induction of *mfa1* was observed in the presence of 6 mM cAMP, but the repression at higher concentrations was impeded. These results can be explained only by assuming that cAMP exerts two separate functions on *prf1*: at lower concentrations, *prf1* is activated at a post-transcriptional level, whereas at higher concentrations, transcriptional repression occurs.

One of the major targets of cAMP in eukaryotic cells is the PKA, a tetramer composed of two regulatory and two catalytic subunits (Taylor et al., 1990). After cAMP binding to the regulatory subunits, the catalytic subunits are released and phosphorylate target proteins. In *U. maydis*, two catalytic (Adr1 and Uka1) and one regulatory subunit (Ubc1) of PKA have been identified (Gold et al., 1994; Orth et al., 1995; Dürrenberger et al., 1998). To investigate whether the divergent effects of cAMP are mediated by these kinases, we monitored *mfa1* gene transcription in a FB1Δubc1 strain after cAMP stimulation (Figure 3). In this mutant, the Adr1 kinase is constitutively activated, as shown by Dürrenberger et al. (1998). This in turn leads to high *mfa1* expression in the absence of external cAMP. Surprisingly, we observed that the repression by cAMP still was functional in the *ubc1* mutant strain. These results show that Ubc1 is involved in the activation of Prf1 and that a second cAMP-regulated pathway that mediates the transcriptional repression must exist.

DISCUSSION

There are two major functions of Prf1 during the development of *U. maydis*: one is to provide high levels of pheromone and receptor gene transcription during mating, and the other is to maintain expression of the *b* genes during pathogenic development. In this study, we have shown that multiple signals are integrated via Prf1 at the transcriptional as well as the post-transcriptional levels.

A detailed analysis of the genetic and environmental control of *prf1* gene activity revealed at least four different signals affecting *prf1* gene expression: (1) carbon source, (2) pheromone stimulation, (3) the b heterodimer, and (4) two separable effects of the cAMP pathway. Carbon source and the cAMP signal regulate *prf1* transcription via a newly identified upstream regulatory element (UAS). Furthermore, we have identified a protein (Ncp1) that binds to the UAS but appears not to be involved in the regulation of *prf1* by these signals.

Transcriptional and post-transcriptional control levels could be distinguished by fusing the *prf1* gene to a constitutive promoter. The constitutive expression of *prf1* did not interfere with sexual development of the fungus; for example, the strains were fertile, able to induce tumors, and developed viable teliospores in the plant.

By using promoter analysis, we identified a UAS that is essential for *prf1* gene activity and accounts for the media control. Further experiments revealed that the UAS mediates gene activation in response to various carbon sources. We suggest that it is not the metabolic state of the cell but the sensing of certain sugars that leads to gene induction via the UAS. Strikingly, the presence of glucose and sucrose leads to induction, whereas maltose does not, although it supports growth as well as does glucose. In yeast, sucrose is cleaved extracellularly by the secreted enzyme invertase, and the sugar monomers are transported into the cell by hexose transporters. On the contrary, maltose is taken up as a disaccharide by a distinct transporter (Carlson, 1987). Two of the hexose transporters have been implicated in signaling functions for the control of glucose-induced genes (Özcan et al., 1996, 1998; Klein et al., 1998; Johnston, 1999). A similar pathway seems to exist in *Neurospora crassa.* In this fungus, the sugar transporter rco-3 is involved in regulation of conidiation (Madi et al., 1997). Therefore, it is conceivable that transcription of *prf1* is regulated through a similar signal transduction pathway, as shown in Figure 7.

Another signal that acts on the UAS is transmitted via the cAMP pathway. We have shown that the UAS activity decreases at high cAMP concentrations. Because carbon metabolism is known to influence cAMP synthesis, this could indicate that the UAS responds to a signal via cAMP and an independent glucose signal or that both effects may be channeled through the cAMP pathway (Figure 7; Eraso and Gancedo, 1985; Thevelein, 1994). The Ncp1 protein, which has been identified as a potential regulator of *prf1*, constitutes a novel DNA binding protein that contains none of the previously characterized DNA binding motifs or any other homology to known proteins. Ncp1 seems to be nonessential for the regulation by carbon source or by cAMP because *ncp1* mutants show normal *mfa1* gene transcription. However, the induction by carbon source is reduced in the *ncp1* mutant strain and appears increased in the strain overexpressing *ncp1.* This may indicate the presence of a functionally redundant protein in *U. maydis.* Alternatively, Ncp1 might respond to a signal that was not investigated here. In any case, the proposed activator of *prf1*, which mediates the effects described above, escaped detection via the gel shift assay.

Post-transcriptionally, *prf1* is activated by the pheromone and cAMP pathways, because in strains expressing *prf1* constitutively, the target gene *mfa1* still is regulated by these

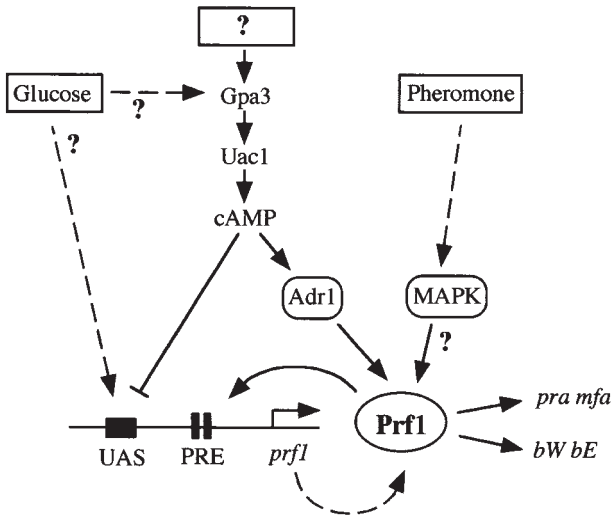

Figure 7. Model of the Signal Transduction Pathways Regulating *prf1* Activity.

All aspects of this model are discussed in the text. Question marks indicate hypothetical interactions. Dashed arrows indicate that the respective signaling components remain to be identified.

signals. Although we cannot exclude the possibility that these signals act on another protein that, in concert with Prf1, controls *mfa1* gene expression, we consider it most likely that they regulate Prf1 protein activity. In yeast, the pheromone signal is transmitted by a heterotrimeric G protein and a mitogen-activated protein (MAP) kinase cascade leading to phosphorylation and activation of the transcription factor Ste12 (Song et al., 1991; Oehlen and Cross, 1997; Pi et al., 1997). The primary amino acid sequence of Prf1 contains five potential phosphorylation sites for MAP kinases (Hartmann et al., 1996), suggesting that a similar mechanism could exist in *U. maydis* (Figure 7). Attempts to demonstrate this directly have failed, because Prf1 proved highly unstable in protein extracts of *U. maydis* (H.A. Hartmann, unpublished data).

We have now provided evidence that not only the pheromone signal but also the positive effect of cAMP affect Prf1 post-transcriptionally, as depicted in Figure 7. Aside from MAP kinase target sites, the amino acid sequence of Prf1 contains five potential phosphorylation sites for cAMP-dependent protein kinases (Taylor et al., 1990), and a phosphorylation of Prf1 by Adr1 currently is being investigated.

A second function of cAMP on *prf1* was revealed by cAMP feeding experiments. Whereas the addition of intermediate amounts of cAMP led to an increase in *mfa1* transcripts, higher concentrations led to repression. The repressing effect was independent of the post-transcriptional activation of Prf1 because it could not be seen in strains in which transcription of *prf1* was constitutive. To explain these effects, we propose the existence of two independent cAMP pathways, each responding to different cAMP levels. We already have speculated that Prf1 phosphorylation is mediated by the Adr1 kinase (Figure 7). The transcriptional repression could be governed by another catalytic subunit of PKA. This kinase should interact with a regulatory subunit different from Ubc1, because the transcriptional repression of *mfa* by high cAMP levels is still functional in a Δubc1 strain. Alternatively, the repressing effect observed at high cAMP concentrations might reflect a PKA independent mechanism of gene regulation.

Recently, divergent cAMP signaling pathways have been proposed to control growth and pathogenicity of the rice blast fungus *Magnaporthe grisea* (Adachi and Hamer, 1998). These results, including the ones presented here, fit the common scheme of cAMP being an important second messenger during fungal pathogenesis (Kronstad, 1997). The intricate control of *prf1* activity in *U. maydis* may have evolved to channel various signals from the environment into distinct cellular responses, such as the decision to grow as yeast, engage in mating, or colonize a maize plant.

METHODS

Strains and Growth Conditions

The *Escherichia coli* K-12 derivative DH5α (Bethesda Research Laboratories) was used for cloning purposes. Strains of *Ustilago maydis* used in this study are listed in Table 2. HA282 was isolated from a cross of FB1 and HA263. Cells were grown at 28°C in YEPS (1% yeast extract, 0.4% trypton, and 0.4% sucrose; modified after Tsukuda et al., 1988) or PD medium (Difco). Minimal medium (Holliday, 1974) contained 1% glucose and 30 mM KNO_3, unless indicated otherwise. cAMP (Sigma) was added directly to the medium, which was filter-sterilized before use. To test for mating and pheromone stimulation, we co-spotted strains on charcoal-containing PD plates and incubated them at room temperature for 48 hr. Plant infections were performed as described in Gillissen et al. (1992).

DNA and RNA Procedures

U. maydis DNA was prepared according to Hoffman and Winston (1987). For radioactive labeling of DNA, the Megaprime DNA labeling kit (Amersham) was used. DNA sequencing was conducted using an ABI 373 automated sequencer (Perkin-Elmer, Foster City, CA). Transformation of *U. maydis* followed the protocol of Schulz et al. (1990). RNA was isolated from strains grown in liquid and solid media by using protocols of Schmitt et al. (1990) and Timberlake (1986), respectively. Magnetic beads (Dynal, Oslo, Norway) facilitated selection of poly(A)$^+$ RNA. All other molecular techniques followed standard procedures (Sambrook et al., 1989). The following probes were used for RNA gel blot analysis. A 680-bp EcoRV fragment isolated from pUMa1 (Bölker et al., 1992) was used to probe for *mfa1*, and a 1.6-kb EcoRV fragment from pRF–6.0B was used to probe for *prf1*. To standardize loading, we used a 1.2-kb actin1 cDNA fragment from *U. maydis* (H.A. Hartmann, unpublished data). Detection and quantification of the signals were performed with the help of a

Table 2. Strains of *U. maydis* Used in This Study

Strain	Relevant Genotype	Reference
FB1Δuac1	*a1 b1 Δuac1*	Krüger et al. (1998)
CL13	*a1 bW2 bE1*	Bölker et al. (1995a)
FB1	*a1 b1*	Banuett and Herskowitz (1989)
FB1Δubc1	*a1 b1 Δubc1–nat*	S. Grüneis and R. Kahmann, unpublished data
FB2	*a2 b2*	Banuett and Herskowitz (1989)
FB6b/pra2	*a1 b2 pra2*	Bölker et al. (1992)
FBD12-17	*a2 a2 b1 b2*	Banuett and Herskowitz (1989)
HA87	*a2 b2 Δprf1-1*	Hartmann (1997)
HA99	*a1 b1 Δprf1-1*	Hartmann (1997)
HA185	*a2 b2 B-UAS3*	This study
HA232	*a2 b2 B-UAS3/cbx*	This study
HA263	*a2 b2 B-UAS3/cbx Δncp1*	This study
HA266	*a2 b2 prf1^{con}*	This study
HA269	*a1 bW2 bE1 Δncp1*	This study
HA271	*a1 b1 prf1^{con}*	This study
HA284	*a2 b1 prf1^{con}*	This study
HA282	*a1 b1 Δncp1*	This study
HA296	*a1 b1 ncp1^{otef}*	This study

STORM PhosphorImager and the ImageQuant program (Molecular Dynamics, Sunnyvale, CA).

Plasmids and Plasmid Constructs

For subcloning and sequencing, plasmids pBluescript II SK+ (Stratagene, La Jolla, CA), and pSP72 (Promega) were used. pCM54 and pSS1 are vectors for transformation of *U. maydis.* They are both capable of autonomous replication and confer resistance against hygromycin and carboxin, respectively (Tsukuda et al., 1988; Keon et al., 1991, Schauwecker et al., 1995). In pSP–Hyg, the hygromycin resistance cassette, which was isolated as a 3.2-kb PvuII fragment from pCM54, was ligated into the SmaI site of pSP72. pSP–cbx is a pSP72 derivative, carrying the *cbx* gene from pSS1, cloned as an EcoRI-EcoRV fragment.

Plasmid p123 (C. Aichinger and R. Kahmann, unpublished data) contains the carboxin resistance gene and a translational fusion of the *egfp* gene (Clontech, Palo Alto, CA) to the *otef* promoter (Spellig et al., 1996). pRF–4.7H and pRF–6.0B are pSP72 derivatives, harboring genomic 4.7-kb HindIII and 6.0-kb BamHI fragments of the *prf1* locus, respectively (Hartmann, 1997). pUT–tef (Hartmann, 1997) is a pUC19 derivative containing the *U. maydis tef1* promoter as an EcoRI-BamHI fragment. A cDNA library was constructed in phage λ ZAPII, by using RNA isolated from strain FB1, which had been grown in YEPS to an $OD_{600\,nm}$ of 1.0. cDNA synthesis, and cloning were performed using the Uni-ZAPII-cDNA Synthesis Kit (Stratagene).

To generate plasmids for promoter analysis, we first cloned the *prf1* gene as 4.7-kb HindIII fragment from pRF–4.7H into the respective site of pSS1. Deletion of a 1.0-kb EagI-NdeI site in the 3′ region of *prf1* resulted in pRFΔP. To extend the promoter region, a 0.3-kb EcoRI-HindIII fragment and a 1.0-kb SphI-HindIII fragment, the latter being modified with EcoRI linkers, were isolated from pRF–6.0B and ligated into the EcoRI-HindIII sites of pRFΔP, yielding $pRF\Delta P_{1.4}$ and $pRF\Delta P_{2.0}$, respectively. For $pRF\Delta P_{1.6}$, a 0.4-kb XhoI-NdeI fragment from pRF–6.0B was blunt ended and subcloned into the SmaI site of pBluescript II SK+. From this plasmid, a 0.2-kb EcoRI fragment was inserted into the respective site of $pRF\Delta P_{1.4}$.

Nested deletions were generated according to the protocol of Ausubel et al. (1987), starting from $pRF\Delta P_{1.6}$, which was linearized at the PstI site. After Bal31 treatment, the DNA was digested with SmaI and subcloned into the SmaI site of pBluescript II SK+. After determination of the deletion end points, promoter fragments of selected clones were excised using HindIII and inserted into the respective site of pRFΔP. Synthetic oligonucleotides comprising the upstream activating sequence (UAS) were R1 (5′-CACACTATACGTTGGTGTGGTCTTGC-3′) and N2 (5′-GTCGACGGTGTGCATATTTGCAAGACCACAC-3′), which were annealed at 20°C and extended by using the Klenow fragment of DNA polymerase I. For the *mfa–sgfp* reporter gene construct, the *mfa1* basal promoter was amplified from pUMa1 by using primers CA1 (5′-GGATCCGTCACCTTCCTGGATGAG-3′) and CA2 (5′-CCATGGTTTCGATCTTCGCTCAG-3′) and cloned together with the *sgfp* gene, which was isolated as an NcoI-SmaI fragment from plasmid blue–SGFP–TYG–nos SK (Sheen et al., 1995) into the BamHI and SmaI sites of pSP72. The *mfa1–sgfp* fusion construct was excised from there using the same enzymes and inserted together with the hygromycin resistance cassette, which was isolated as 2.8-kb PvuII-HindIII fragment from pCM54 into the HindIII-BamHI sites of pSP72, resulting in pmfa–sgfp. From one of the nested deletions, an 85-bp XhoI-SalI fragment, comprising the UAS, was isolated and ligated into the XhoI site of pBluescript II SK+ in different copy number and orientation. From these, the UAS were excised as XhoI-SalI fragments and ligated into the BamHI or EcoRV sites of pmfa–sgfp, resulting in pE–UAS, pE–UASi, pB–UAS, pB–UAS2i, and pB–UAS3.

Constructs for Gene Replacements

The *prf1^{con}* allele was generated from pRFΔP by inserting a 250-bp EcoRI-NdeI fragment from pUT–tef, comprising the *tef1* promoter, and a 0.6-kb NdeI-MluI fragment from pET–prf (Hartmann et al., 1996), encoding the N-terminal half of Prf1, into the EcoRI-MluI sites. To enable gene replacement, we inserted a 1.5-kb EcoRI-SalI fragment from $pRF\Delta P_{1.4}$ into the blunt-ended EcoRI site. Plasmid pRF^{con} was linearized with HindIII before transformation.

A 3.6-kb EcoRI fragment comprising the *ubc1* gene was isolated from a cosmid library and cloned into pTZ19R. A 1.5-kb BssHII fragment, comprising most of the *ubc1* reading frame, was replaced by a 1.4-kb AscI NAT resistance cassete from pNEB Nat(−) (A. Brachmann and R. Kahmann, unpublished data), resulting in pΔubc1–nat (S. Grüneis and R. Kahmann, unpublished data).

For the *ncp1* deletion construct, a 1.7-kb BamHI fragment from pNcp–7.0H was cloned into the respective site of pSP72–Hyg, and a 1.2-kb BglII-PstI fragment was ligated into the BamHI-PstI sites of pSP72. From the former, a 4.9-kb HindIII fragment carrying the 3′ region of *ncp1* and the hygromycin resistance cassette were excised and ligated into the HindIII site of the latter construct. Plasmid pΔncp1 was linearized with EcoRV and transformed into strains CL13 and HA232 of *U. maydis.* Overexpression of *ncp1* was achieved by cloning a 1.7-kb EcoRI-BamHI fragment from pNcp–7.0H, comprising the transcribed region of *ncp1* into the EcoRI and HpaI sites of p123. Plasmid pncp1^{otef} was linearized within the *cbx*

resistance gene with SspI and transformed into strain FB1. Integration at the *cbx* locus and gene replacements were confirmed by DNA gel blot analysis.

Gel Retardation Analysis and Purification of the Ncp1 Protein

DNA fragments used in the gel-retardation assays were excised from pmfa–sgfp and pUAS–sgfp as BglII-AvaI fragments and end labeled with α-^{32}P-dCTP by using the Klenow fragment. Gel retardation assays were performed as described previously (An et al., 1997) by using the following DNA binding buffer: 20 mM Tris-HCl, pH 7.9, 0.1 M NaCl, 0.5 mM EDTA or 3 mM $MgCl_2$, 1 mM DTT, 0.05% (v/v) Triton X-100, 100 μg mL^{-1} salmon testes DNA, and 15% (v/v) glycerol. Crude protein extracts of *U. maydis* were prepared as described in Basse et al. (1996) and dialyzed against buffer H (20 mM Tris-HCl, pH 7.9, 0.5 mM EDTA, 0.1 M NaCl, 0.05% [v/v] Triton X-100, 1 mM DTT, 0.2 mM Pefabloc [Boehringer Mannheim], and 15% [v/v] glycerol). Twenty milliliters of protein extract was loaded on a 5-mL heparin–Sepharose column (Bio-Rad) at a flow rate of 1 mL min^{-1} and washed with buffer H until protein was no longer detected in the flow-through by measuring the relative absorbance at 280 nm. Proteins were eluted by applying a linear gradient from 0.1 to 1.0 M NaCl over 40 min with a constant flow rate of 1 mL min^{-1}.

Protein fractions were tested in a gel retardation assay, and active fractions were pooled and desalted using a PD-10 column (Pharmacia), eluting with buffer H. This protein preparation was purified further on a Mono Q anion exchange column (1 mL; Pharmacia) by using a linear gradient from 0.1 to 0.6 M NaCl for elution. Protein fractions with UAS binding activity were concentrated by ultrafiltration (Nanosep 30K; Filtron, Northborough, MA) and separated on a 12% SDS–polyacrylamide gel. Two candidate proteins with a molecular mass of $\sim$60 kD where identified, which are present in all active fractions. For both proteins, peptide sequences of a Lys-C digest were obtained after the protocol of Eckerskorn and Lottspeich (1989).

Isolation of the *ncp1* Gene

From one of the peptide sequences, the degenerate primer pep3c (5′-CARGCNCGNTCNCGNCARGC-3′; N: A/T/C/G; R: A/G) was designed and used together with the T7 primer (5′-GAATTGTAATACGACTCACTATAG-3′) in a polymerase chain reaction. Thirty nanograms of a plasmid library, which was generated by using the ExAssist/SOLR system (Stratagene) from $\sim 1 \times 10^9$ cDNA clones, served as the template. Reactions contained 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM deoxynucleotide triphosphates, and 50 pmol of primers. The following program was used for amplification in a StratageneRobocycler: 2 min at 94°C and 30 cycles of 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C, with final extension for 8 min at 72°C. Selected fragments were isolated, reamplified, and cloned using the TOPO TA cloning kit (Invitrogen, Leek, The Netherlands). A cloned 420-bp fragment was used to screen both a cosmid library (Bölker et al., 1995b) and the original cDNA library. From cosmid 28G8, a 7-kb HindIII fragment was subcloned into pSP72, resulting in pNcp–7.0H.

Fluorometric Determination of Fluorescence Intensity

Fluorescence intensity was determined using a SFM 25 fluorometer (Kontron Instruments, Zürich, Switzerland), as described by Spellig et al. (1996).

Nucleotide Sequence Accession Number

The nucleotide sequence of the *ncp1* gene has the GenBank accession number AF082296, and *prf1* has the accession number U40753. The *prf1* sequence has been updated to include the full promoter sequence.

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